Electromagnetic Properties of Hemoproteins

V. OPTICAL AND ELECTRON PARAMAGNETIC RESONANCE CHARACTERISTICS OF NITRIC OXIDE DERIVATIVES OF METALLOPORPHYRIN-APOHEMOPROTEIN COMPLEXES*

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SUMMARY

Paramagnetic complexes of iron (III), manganese (II), and cobalt (II) protoporphyrins IX and apohemoproteins react with nitric oxide to form spectroscopically distinct compounds. Optical and electron paramagnetic resonance (EPR) studies suggest that a 1-electron transfer between nitric oxide and metal ions which occurs in these reactions results in the formation of spin-paired complexes of these metalloporphyrins with nitric oxide. However, manganese (III) and cobalt (III) protoporphyrin-apohemoprotein complexes do not react with nitric oxide.

Ferrous hemoproteins react reversibly with nitric oxide to form paramagnetic complexes, which exhibit an intense EPR absorption centered around \( g = 2.0 \). The complexes of nitric oxide with ferrous cytochrome \( c \) peroxidase and horseradish peroxidase show well defined EPR absorptions with rhombic symmetry \((g_1 = 2.08, g_2 = 1.96, \text{ and } g_3 = 2.004)\). The hyperfine structure in the \( c \) absorption has been analyzed through isotope substitutions with \( ^{15} \text{N} \) and \( ^{57} \text{Fe} \)-enriched heme. It is concluded that the unpaired electron of nitric oxide with ferrous cytochrome \( c \) peroxidase and horse-radish peroxidase show well defined EPR absorptions with EPR parameters of nitric oxide complexes of ferrous hemoproteins are not significantly affected by substitutions of two vinyl groups (protoheme) at positions 2 and 4 of the porphyrin ring with two ethyl groups (mesoheme) or hydrogens (deuteroheme), indicating that the nucleophilicity of porphyrin side chains has no appreciable effect on the metal-ligand bond strength.

The elucidation of the physical and chemical nature of the heme environment provided by the protein is of primary importance in our understanding of the diversity of reactivity and function of hemoproteins containing identical or closely related prosthetic groups. The recent determination of the three-dimensional molecular structures of myoglobin, hemoglobin, and cytochrome \( c \) by x-ray crystallography (2-4) has made possible the examination of the details of the heme environment at atomic levels. The heme ligands have been unequivocally established and the amino acid residues which form the hydrophobic heme cleft have been identified in these hemoproteins. On the other hand, structural information on other hemoproteins, particularly heme-enzymes such as the peroxidases, catalases, and oxidases, is quite limited, despite the extensive mechanistic studies which have been made on these enzymes. Even the heme ligands of these enzymes are yet to be identified, although carboxylate anion (5, 6) amino groups (6) and imidazole groups (6) have been postulated as possible heme ligands in horseradish peroxidase and catalase.

Application of extrinsic spectroscopic probes to hemoproteins has yielded valuable information regarding the heme environment. For example, the hydrophobic nature of the heme cleft (7) has been confirmed by use of fluorescence probes (8-10) as well as by x-ray crystallographic studies (2-4). Conformational changes in the vicinity of the heme group upon ligand binding have been demonstrated by the use of spin labeled probes attached to apoprotein (11) and heme (12). Nitric oxide is a heme ligand which contains 1 unpaired electron and is used here as a "spin-labeled ligand" for probing the electronic structure of the heme group and the heme environment. An electron paramagnetic resonance signal was first detected in a NO-hemoglobin by Bennett et al. (13). EPR spectra of NO-hemoglobin have been analyzed (14-20) as a model of diamagnetic oxy- and carbon...
Nitric oxide reacts with both ferrous and ferric hemoproteins (21–25). We have examined optical and EPR characteristics of the nitric oxide complexes of heme-enzymes including horseradish peroxidase, cytochrome c peroxidase, and catalase as well as other metalloporphyrin-apohemoprotein complexes (26, 27) in order to identify the heme ligand in these enzymes and to probe the spin pairing phenomena between nitric oxide and paramagnetic metal ions in these metalloporphyrin-apohemoprotein complexes.

**EXPERIMENTAL PROCEDURE**

**Materials**—Cytochrome c peroxidase was purified from bakers' yeast as described previously (28, 29). Horseradish peroxidase (Sigma) and horse liver catalase (Boehringer) were purchased and purified by ion exchange column chromatography. Hemoglobin was purified from human blood according to the method of Drabkin (30). Myoglobin was purified from skeletal muscle of sperm whale by the method of Kendrew and Parrish (31). The $^{57}$Fe-enriched protoheme was prepared by incorporating 90% $^{57}$Fe-enriched iron into protoporphyrin IX (32). Meso-

![Fig. 1. Optical absorption spectra of ferric and ferrous cytochrome c peroxidase (CCP) and horseradish peroxidase (HRP) and their compounds.](http://www.jbc.org/content/247/8/2448/F1)

Nitric oxide was prepared by the method of Drabkin (30). Myoglobin was purified from skeletal muscle of sperm whale by the method of Kendrew and Parrish (31). The $^{57}$Fe-enriched protoheme was prepared by incorporating 90% $^{57}$Fe-enriched iron into protoporphyrin IX (32). Meso-

heme, deuteroheme, and manganese protoporphyrin IX were prepared as described previously (32, 33). Cobalt protoporphyrin IX was synthesized by the method of Falk (34). The metalloporphyrins were recombined with apohemoproteins as described previously (32, 33). The recombined products were purified by ion exchange column chromatography. Nitric oxide (Matheson) was washed with 1 M KOH and anaerobically added to samples. Sodium nitrite (Baker) and $^{15}$N-labeled sodium nitrite (Thompson-Packard) were used without further purification. The concentrations of hemoproteins were determined spectrophotometrically by the pyridine hemochromogen procedure. The concentrations of manganese- and cobalt-porphyrin-protein complexes were determined spectrophotometrically using appropriate extinction coefficients (26, 33). The concentrations referred to are metal ion concentrations.

**Methods**—The combination of ferric and ferrous hemoproteins with nitric oxide was carried out anaerobically in a Thunberg cuvette. Prepurified helium (Matheson) was passed over the surface of the solution for 30 min with occasional stirring, and then was replaced with nitric oxide which was passed over the surface of the solution for 5 to 10 min. If the protein was stable, the sample was repeatedly evacuated and flushed with prepurified helium, before the introduction of nitric oxide. In addition, the nitric oxide complexes of ferrous hemoproteins were prepared by addition of a few milligrams of sodium nitrite and sodium dithionite to solutions of ferric hemoproteins. This technique was convenient, because oxygen was removed by the excess dithionite and no precaution was necessary to maintain the...
solution anaerobic. The nitric oxide compounds formed were anaerobically transferred to EPR sample tubes and immediately frozen in liquid nitrogen. The nitric oxide compound of ferrous catalase was prepared by addition of ethyl hydrogen peroxide to give a final concentration of 1 mM to a solution of 0.4 mM catalase and 1 mM azide. The concentrations of metallocporphyrin-protein complexes were 10 to 50 μM and 200 to 500 μM for optical and EPR measurements, respectively. Potassium phosphate buffer, pH 7.0 (0.1 M), was used unless otherwise stated. Spectrophotometric measurements were carried out with a Cary 15 recording spectrophotometer at 23°. EPR measurements were made with a Varian X- and K-band spectrometer (V-4502 and V-4503) with 100 kHz field modulation. A variable temperature assembly was employed for measurements at low temperatures using liquid nitrogen and liquid helium as coolant. A chloroform solution of 2,2-diphenyl-1-picrylhydrazyl (0.1 to 0.5 mM) was used for the g value calibration. EPR spectra were simulated with a PDP-6 digital computer in order to estimate values of hyperfine coupling constants when poor resolution prevented their direct determination.

**TABLE I**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Absorption maxima</th>
<th>Reference</th>
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<tr>
<td>Ferric CCP-NO</td>
<td>419 120 537 12.5 571 16.0</td>
<td>This paper</td>
</tr>
<tr>
<td>Ferric HRP-NO</td>
<td>419 144 533 13.0 568 16.0</td>
<td>This paper</td>
</tr>
<tr>
<td>423 539 570</td>
<td>21</td>
<td></td>
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<tr>
<td>533 12.1 568 14.8</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Ferrous CCP-NO</td>
<td>421 99 542 12.0 572 11.0</td>
<td>This paper</td>
</tr>
<tr>
<td>Ferrous HRP-NO</td>
<td>421 110 542 11.5 570 10.5</td>
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<tr>
<td>423.5 545</td>
<td>9.8</td>
<td>24</td>
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**RESULTS**

*Nitric Oxide Ferric Peroxidases*—Cytochrome c peroxidase and horseradish peroxidase rapidly formed spectrally distinct complexes upon anaerobic addition of nitric oxide. The nitric oxide complexes were stable: no spectral change was observed on allowing them to stand anaerobically at pH 5 to 10 for 10 hours at 296°K. In contrast, the nitric oxide complexes of other ferric hemoproteins such as hemoglobin (24, 25) and myoglobin (22, 24, 25) were reported to be unstable and gradually transformed to the corresponding ferrous nitric oxide complexes. The binding of nitric oxide with ferric peroxidases was fully reversible. The original ferric peroxidases were readily recovered by degassing the nitric oxide ferric derivatives under reduced pressure. Their absorption spectra were characterized by intense α- and β-bands (cf. Fig. 1 and Table I). The shapes and positions of these bands were typical of a low spin hemochromogen, although the original peroxidases were in high spin ferric states. Low temperature EPR examination revealed that the EPR absorption of ferric heme iron in the original peroxidases had disappeared completely upon the formation of nitric oxide compounds.
and no new EPR absorption was detected in a temperature range of 4.2-296°K.

**Nitric Oxide Manganese-Porphyrin Complexes**—Manganese (III) protoporphyrin-containing peroxidases, hemoglobin, and myoglobin did not react with nitric oxide. However, their reduced forms containing manganese(II) protoporphyrin formed spectrally distinct complexes with nitric oxide (Fig. 2). The over-all shapes and positions of absorption bands were strikingly similar to those of the nitric oxide ferric peroxidases mentioned above. The axially symmetric EPR signal of high spin manganese(II) protoporphyrins (g_L = 5.9 and g_l = 2.0) disappeared in these nitric oxide complexes (Fig. 3).

**Nitric Oxide Cobalt-Porphyrin Hemoglobin**—As reported recently by Hoffman and Petering (27), cobalt(II)-containing hemoglobin exhibits an EPR spectrum of axial symmetry (g_L = 2.36 and g_l = 2.08) with well resolved hyperfine splittings due to 57Co and proximal 14N nuclei. The oxygenation of cobalt(II)-containing hemoglobin changed the EPR signal to one with axial symmetry (g_L = 2.00 and g_l = 2.09), which was attributed to a superoxide anion bound to the metal (27). Upon deoxygenation, the initial EPR spectrum could be restored. The EPR signal of cobalt(II)-containing hemoglobin disappeared completely upon reaction with nitric oxide.

**Nitric Oxide Ferrous Peroxidases** The dithionite-reduced ferrous horseradish and cytochrome c peroxidases reacted rapidly with nitric oxide to form the corresponding nitric oxide peroxydases. The nitric oxide peroxidases exhibited an intense EPR absorption centered around g = 2 with rhombic symmetry (Figs. 4A and 5C). The central resonance signal at g = 2.004 (Table II) exhibited a well resolved hyperfine structure of a triplet of triplets. When nitric oxide was substituted with 15N0, the hyperfine structure became a doublet of triplets (Fig. 5A). Upon the combination of 57Fe-enriched ferrous peroxidases with either 15NO or 14N0 eight or ten hyperfine lines, respectively, were observed in the central resonance signal (Figs. 4A and 5C). The line shapes of the two signals at low and high fields were also affected by these isotope substitutions. However, the hyperfine structure in these two resonance signals was not well resolved even at K-band (Fig. 6). The second derivative display indicated that the low and high field signals had a triplet structure (Fig. 7).

The EPR spectra of nitric oxide ferrous horseradish and cytochrome c peroxidases containing meso- and deuterohemes were identical with those of the corresponding protoheme-containing compounds.
Table II

Effect of isotope substitution on electron paramagnetic resonance parameters of ferrous hemoprotein nitric oxides

<table>
<thead>
<tr>
<th>Type</th>
<th>Compound*</th>
<th>Principal g value</th>
<th>Hyperfine coupling constant in the axial direction</th>
<th>Number of hyperfine lines</th>
<th>Reference</th>
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<td>Axial (g_{zz})</td>
<td>In-plane (g_{zz})</td>
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<td></td>
<td></td>
<td>2.080</td>
<td>2.004</td>
<td>1.955</td>
<td>30</td>
</tr>
<tr>
<td></td>
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<td>2.080</td>
<td>2.004</td>
<td>1.955</td>
<td>30</td>
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<td></td>
<td>HRP-^{13}NO</td>
<td>2.080</td>
<td>2.004</td>
<td>1.955</td>
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<td></td>
<td>CCP-^{14}NO</td>
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<td>2.004</td>
<td>1.960</td>
<td>30</td>
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<tr>
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<td>CCP-^{14}NO</td>
<td>2.080</td>
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<td>1.960</td>
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<tr>
<td></td>
<td>CCP-^{14}NO</td>
<td>2.080</td>
<td>2.004</td>
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<td>Hb-^{14}NO</td>
<td>2.070</td>
<td>2.004</td>
<td>1.955</td>
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<td></td>
<td>Hb-^{14}NO</td>
<td>2.070</td>
<td>2.004</td>
<td>1.955</td>
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<td>2.004</td>
<td>2.00</td>
<td>22.4</td>
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</table>

* CCP, cytochrome c peroxidase; HRP, horseradish peroxidase; LP, lactoperoxidase; and ^{57}CCP and ^{8}HRP, CCP and HRP containing ^{57}Fe-enriched protoporphyrin.

Fig. 6. X- and K-band EPR spectra of ^{15}NO-ferrous horseradish peroxidase containing ^{57}Fe (^{57}HRP-^{15}NO) at 77°K.

Fig. 7. X-Band EPR spectra of ^{15}NO-ferrous horseradish peroxidase at 77°K. A, first derivative display; B, second derivative display.

Nitric Oxide Derivatives of Other Ferrous Hemoproteins—Nitric oxide derivatives of ferrous catalase, cytochrome c (23), myoglobin (36), and hemoglobin (17–20) also showed an EPR absorption centered around g = 2 with rhombic symmetry. However, the line shapes of the EPR signal of these compounds were
complex because of partial overlap between signals from the three principal \( g \) values and the width of the individual signals (Fig. 4, B, C, and D). The resolution of EPR spectra of these compounds was significantly improved by the substitution of \(^{15}\text{N}0\) with \(^{14}\text{N}0\) (Fig. 4, C and D) and by EPR measurements at K-band (Fig. 8). Thus it was possible to assign the three principal \( g \) values (Table II) and the hyperfine structure of the central EPR signal.

The principal \( g \) values and hyperfine coupling constants of nitric oxide ferrous hemoglobin and myoglobin were not significantly affected by substitution of protoheme with meso- or deuteroheme.

**DISCUSSION**

**Nature of Nitric Oxide Complexes of Iron(III), Manganese (II), and Cobalt(II) Protoporphyrin Compounds**—The fact that the nitric oxide compounds of ferric peroxidase are EPR-negative and that their optical spectra are of a low spin hemochromogen type supports the proposal of Ehrenberg and Szczepkowski (22) that the interaction of ferric hemoproteins with nitric oxide is accompanied by the transfer of electrons from nitric oxide to the ferric heme iron. This electron transfer results in the formation of an EPR-negative complex, as schematically illustrated in Fig. 9A. The nitric oxide complexes of ferric myoglobin and hemoglobin (22, 24, 25) are reduced to the corresponding nitric oxide ferrous compounds on standing in the presence of nitric oxide. The reduction of the ferric heme by nitric oxide occurs more rapidly in hemoglobin than in myoglobin. The nitric oxide complexes of ferric peroxidases, on the other hand, are not reduced by nitric oxide under comparable conditions (pH 5 to 10). The order of reducibility of the heme iron by nitric oxide appears to be related to the order of oxidation-reduction potential of these hemoproteins (36-39).

The manganese(II) protoporphyrin-apohemoprotein complexes are \( Sd^1 (S = 5/2) \) systems which are isoelectronic with high spin ferric hemoproteins. The absence of EPR signals for nitric oxide manganese(II) compounds and the close resemblance of their optical absorption spectra to nitric oxide ferrie hemoproteins suggest that the transfer of electrons from nitric oxide to the metal may have taken place in these compounds to form an EPR-negative complex (Fig. 9B). The loss of the EPR absorption of cobalt(II)-containing hemoglobin upon reaction with nitric oxide can also be interpreted as a spin pairing or charge-transfer between the metal and the liganded nitric oxide (Fig. 9C). At present, it cannot be determined whether the cobalt ion acts as an electron donor or acceptor. Infrared spectroscopy may be useful to assign structures to these nitric oxide compounds, since \(^{15}\text{N}0\), \(^{14}\text{N}0\), and \(^{14}\text{O}0\) can be distinguished by their infrared stretching frequencies.

**Electron Paramagnetic Resonance Characteristics of Nitric Oxide Derivatives of Ferrous Hemoproteins**—The X-band EPR spectra of nitric oxide ferrous peroxidases (Figs. 4 and 5) clearly indicate that the paramagnetic center of these compounds is a low spin hemochromogen type which supports the proposal of Ehrenberg and Szczepkowski (22) that the interaction of ferric hemoproteins with nitric oxide is accompanied by the transfer of electrons from nitric oxide to the ferric heme iron. This electron transfer results in the formation of an EPR-negative complex, as schematically illustrated in Fig. 9A. The nitric oxide complexes of ferric myoglobin and hemoglobin (22, 24, 25) are reduced to the corresponding nitric oxide ferrous compounds on standing in the presence of nitric oxide. The reduction of the ferric heme by nitric oxide occurs more rapidly in hemoglobin than in myoglobin. The nitric oxide complexes of ferric peroxidases, on the other hand, are not reduced by nitric oxide under comparable conditions (pH 5 to 10). The order of reducibility of the heme iron by nitric oxide appears to be related to the order of oxidation-reduction potential of these hemoproteins (36-39).

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The nitric oxide complexes of ferrous catalase (Fig. 4) and ferrous peroxidase may be the imidazole group of a histidyl residue.

The effect of the $^{57}$Fe isotope substitution on the hyperfine structure in the $z$ absorption of nitric oxide ferrous peroxidase (Fig. 5, B and D) could be adequately explained by assuming the coupling constant of $^{57}$Fe in the $z$ direction ($A_{Fe}$) to be of the same order of magnitude as that of the proximal $^{15}$N ($A_{N}$), resulting in a partial overlapping of the expected hyperfine lines (Fig. 10). Both the observed numbers and relative intensities of the hyperfine lines in $^{57}$Fe-containing compounds were consistent with this explanation. Thus, the $A_{Fe}$ value was estimated to be 6.5 gauss.

As shown in Fig. 5, the shape of the $x$ and $y$ absorptions were definitely affected by the substitutions with $^{15}$NO and $^{57}$Fe. However, the direct determination of coupling constants in these directions from the recorded spectra was not possible because of the poor spectral resolution. The lack of resolution in the $x$ and $y$ absorptions is probably due to inhomogeneous broadening from unresolved dipolar hyperfine coupling to the four pyrrole $^{14}$N nuclei. Interactions with the pyrrole nitrogens would be maximal at those orientations if the unpaired electron is localized in the $a_{g}$ orbital of the iron (18). Spectral simulations of the $x$ and $y$ absorptions for $^{15}$NO-horseradish peroxidase confirmed that the apparent triplet structure of these absorptions arises because the $^{14}$N coupling from the proximal ligand is greater than that from the $^{15}$NO in these directions. The spectral envelope for the $x$ and $y$ absorptions could be reproduced with a line width of ~4.5 gauss (full width at half height), $^{14}$N (proximal ligand) coupling of ~10 gauss, and $^{15}$NO coupling of ~4 gauss. Larger $^{15}$NO coupling in these directions destroys the apparent triplet symmetry of these absorptions in the simulated spectra. Thus, the largest splitting in the central or $z$ absorption arises from the $^{15}$N nucleus whereas the $^{14}$N nucleus of the proximal ligand produces the large splitting in the $x$ and $y$ directions. Since the isotropic coupling constants for $^{15}$NO-horseradish peroxidase cannot be obtained from solution EPR measurements (i.e., the protein tumbles too slowly to average completely the anisotropic contributions) and the relative signs of the $A$ tensor components are unknown, a more detailed analysis of the ligand superhyperfine interactions requires several assumptions. Because of these assumptions, the analysis for $^{15}$NO-horseradish peroxidase (Table III), which was made according to the method of McNeil et al. (40), is only tentative.

These results indicate that the unpaired electron of nitric oxide spends a considerable fraction of the time in the iron orbitals. The direct estimation of the total spin density on the iron requires the knowledge of components of the $A_{Fe}$ tensor ([$A_{Fe}(x,y,z)$]). The in-plane $A_{Fe}$ value ($A_{Fe}(x,y)$) may be estimated from Mössbauer spectra (41). Mössbauer measurements of nitric oxide ferrous peroxidases are under way. The formal structure of nitric oxide ferrous peroxidases, therefore, may be described as shown in Fig. 9D. It is obvious that the electronic configuration of nitric oxide ferrous peroxidase cannot be adequately expressed by assigning a single formal valency state to the heme iron. Instead, we consider delocalization of the unpaired electron as shown in Fig. 9D to be an appropriate expression of the electronic configuration of nitric oxide ferrous peroxidases.

The differences in superhyperfine coupling of heme iron with the nitric oxide ligand and the proximal ligand are striking. With the proximal ligand, the $A_{2}(x,y)$ coupling is greater than the $A_{2}(z)$ coupling. This fact shows that one of the components

* M. Morrison and T. Yonetani, unpublished data.

$^{3}$ G. Lang and T. Yonetani, in preparation.
The tral resolution of Type II compounds is greatly improved at the \( g_z \) values of 2.003 to 2.005, were previously reported for Type II compounds from X-band EPR spectra. Thus \( g_z \) values of 2.00 to 2.01 (Table II), which are larger than \( g_z \) values of Type II compounds from X-band EPR spectra. g values, particularly the \( z \) and \( y \) absorptions, are partially superposed in X-band spectra. Thus, the envelope of its EPR absorption gives an impression of axial rather than rhombic orientation (Fe—NO or Fe—ON), or the protein conformation is currently under investigation.

Although nitric oxide ferrous hemoproteins are classed into two arbitrary groups, there is no qualitative difference between the two groups. The greater separation of the \( z \) and \( y \) absorptions in Type I compounds merely indicates increased in-plane anisotropy of the electronic configuration in peroxidases. Since peroxidases containing nitrogenous and nonnitrogenous groups as the proximal ligand exhibit a set of identical principal \( g \) values, the nature of the proximal ligand is not primarily responsible for the greater in-plane anisotropy in Type I. It is likely that the degree of the \( n-d \) interactions between orbitals of the proximal ligand or nitric oxide, or both, and the heme determines the in-plane anisotropy (18).

It should be mentioned that the substitution of protoheme by mesoheme or deuteroheme had no detectable effect on the EPR characteristics of nitric oxide ferrous hemoproteins. This indicates that the distribution of the unpaired electron in nitric oxide ferrous hemoproteins is insensitive to the changes in the electron-withdrawing character of the porphyrin periphery. In other words, the metal-ligand bond strength in nitric oxide ferrous hemoproteins is insensitive to the changes in the \( \Delta \text{p-d} \) interactions between orbitals of the proximal ligand or nitric oxide, or both, and the heme.

In the Type II compound EPR signals from the three principal \( g \) values, particularly the \( z \) and \( y \) absorptions, are partially superposed in X-band spectra. Thus the envelope of its EPR absorption gives an impression of axial rather than rhombic symmetry. Nitric oxide derivatives of ferrous cytochrome oxidase, cytochrome c peroxidase, and lactoperoxidase belong to Type I.

In the Type II compound EPR signals from the three principal \( g \) values, particularly the \( z \) and \( y \) absorptions, are partially superposed in X-band spectra. Thus the envelope of its EPR absorption gives an impression of axial rather than rhombic symmetry. Nitric oxide derivatives of ferrous cytochrome oxidase (35), catalase, cytochrome c (18, 23), hemoglobin (17, 18), and myoglobin belong to Type II. Because of the spectral overlap, it is difficult to determine accurately the three principal \( g \) values of Type II compounds from X-band EPR spectra. Thus \( g_y \) values of 2.00 to 2.01 (Table II), which are larger than the \( g \) values of 2.003 to 2.005, were previously reported for Type II compounds (17, 18, 20, 23, 35). As shown in Fig. 8, the spectral resolution of Type II compounds is greatly improved at K-band. Thus, the three principal \( g \) values have been accurately estimated from K-band EPR spectra; we found the \( g_y \) value of Type II compounds to be larger than 1.97 and smaller than 1.90 (Table II).

It should be pointed out that an additional EPR signal at \( g = 2.05 \) to 2.03 is consistently observed in Type II compounds except for cytochrome c peroxidase (Fig. 4, B, C, and D). Whether or not this EPR signal at \( g = 2.03 \) indicates the presence of more than one molecular species in these Type II compounds in terms of the Fe—N—O bond angle, the nitric oxide orientation (Fe—NO or Fe—ON), or the protein conformation is currently under investigation.

Although nitric oxide ferrous hemoproteins are classed into two arbitrary groups, there is no qualitative difference between the two groups. The greater separation of the \( z \) and \( y \) absorptions in Type I compounds merely indicates increased in-plane anisotropy of the electronic configuration in peroxidases. Since peroxidases containing nitrogenous and nonnitrogenous groups as the proximal ligand exhibit a set of identical principal \( g \) values, the nature of the proximal ligand is not primarily responsible for the greater in-plane anisotropy in Type I. It is likely that the degree of the \( n-d \) interactions between orbitals of the proximal ligand or nitric oxide, or both, and the heme determines the in-plane anisotropy (18).

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